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Molecular Events During Resting Cyst Formation in Unicellular Eukaryote *Colpoda*

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ABSTRACT

An understanding of intracellular signaling pathways leading to resting cyst formation (encystment) is essential for the development of clinical drugs to prevent the life cycle of pathogenic unicellular eukaryotes. Recent studies imply that there are common signaling pathways among pathogenic and nonpathogenic unicellular eukaryotes. This paper describes molecular events, including signaling pathways, in the encystment of the nonpathogenic unicellular eukaryote *Colpoda*, based on results obtained mainly in our laboratory in the past 20 years.

Abbreviations: Ca^{2+}/CaM: Ca^{2+}/calmodulin; UV: Ultraviolet rays; PKA: Protein kinase A; Phos-tag/ECL: Phosphate-binding tag/enhanced chemiluminescence; LC-MS/MS: Liquid chromatography-tandem mass spectrometry; 2-D PAGE: Two-dimensional polyacrylamide gel electrophoresis; SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis: EF-1α: Elongation factor 1α; AMPK: AMP-activated protein kinase; eEF2K : Eukaryotic Elongation Factor-2 Kinase

Introduction

One strategy of some pathogenic unicellular eukaryotes is to form resting cysts that are resistant to the environmental stresses of the host, such as stomach acid or immune system attack. Therefore, an understanding of intracellular signaling pathways leading to resting cyst formation (encystment) is essential for the development of clinical drugs to prevent cyst formation. It has been reported that the induction of the encystment of pathogenic *Entamoeba histolytica* is Ca^{2+}/calmodulin (Ca^{2+}/CaM)-dependent [1] and cAMP-dependent in the pathogenic unicellular eukaryote *Giardia* [2]. Ca^{2+}/CaM and cAMP are also essential components in intracellular signaling pathways of the nonpathogenic free-living soil ciliate *Colpoda* [3]. These facts imply that common signaling pathways leading to encystment occur in some pathogenic and nonpathogenic unicellular eukaryotes. Recently, early molecular events during the encystment of *Colpoda*, including signaling pathways, were elucidated extensively. In this paper, we describe the encystment process of *Colpoda* based mainly on our research results. In hazardous environments, *Colpoda* promptly transforms into resting cysts that are resistant to desiccation, freezing, high temperature, acid, and UV light [4-7]. In the case of *Colpoda cucullus* Nag-1 [8], encystment can be induced by suspending vegetative *Colpoda* cells at a high cell density in the presence of Ca^{2+} [3].

Morphogenetic Events During Encystment of *C. cucullus* Nag-1

The morphogenetic changes in encystment-induced *Colpoda* cells are described as follows:

2~3 h after Encystment Induction: The cells stop swimming and begin to aggregate by an excretion of sticky mucus. The cells then round up, and then small sticky globules showing a fibrous or crystal-like fine structure [9] called lipidosomes [10] are extruded to be trapped by the mucus layer, followed by the formation of an ectocyst layer (a rigid single-cyst wall layer) [9]. In this stage, the cellular structures characterizing vegetative cells such as cilia begin to disintegrate and mitochondrial activity is arrested [11].

3~5 h after Encystment Induction: At 3~5 h after encystment induction, the first-synthesized endocyst layer is formed by the excretion and gelation of an endocyst-precursor substance between

an ectocyst layer and the plasma membrane [9]. In order to digest vegetative structures, several autophagosomes are formed to digest the vegetative structures.

5 h~1 week after Encystment Induction: The formation of endocyst layers is repeated (presumably twice per day), and several layers of endocyst are formed in several days. Auto phagocytosis is nearly completed within 24h. In the 1-week-old mature cyst, the cytoplasm is filled with an amorphous electron-lucent material and many electron-lucent ellipsoidal granules (presumably reserve grains) accumulate [9].

Signaling Pathways Leading to Encystment and Early Molecular Events During Resting Cyst Formation

Using a pharmacological method, we have proposed that intracellular signaling pathways leading to the encystment of *C. cucullus* Nag-1 are activated by Ca^{2+}-calmodulin, followed by an increase in intracellular cAMP concentrations [3]. Thereafter, an encystment-dependent elevation of the intracellular Ca^{2+} concentration was demonstrated by using Fura 2 ratiometry [12]. Intracellular Ca^{2+} may activate calmodulin, which is thought to activate adenylate cyclase to elevate cAMP, followed by activation of protein kinase A (PKA) [3]. Actually, cAMP enzyme immunoassay (EIA) showed up to ten-fold elevation of cAMP levels in encystment-induced *Colpoda* cells [13,14]. cAMP-dependently phosphorylated proteins were detected by means of biotinylated Phos-tag/enhanced chemiluminescence (ECL) assay [15] and then identified by a liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis [16,17]. The proteins whose phosphorylation levels are up-regulated at 1h after encystment is induced by Ca^{2+}/overpopulation of *Colpoda* vegetative cells or by the addition of membrane-permeation-type cAMP acetoxymethyl ester (cAMP-AM) are listed as follows (most phosphorylated proteins are common in Ca^{2+}/overpopulation induction and cAMP induction):

a. Ribosomal P0 protein (localized in macronucleus)

Presumed function: Regulation of gene expression and metabolism

b. Ribosomal S5 protein

Presumed function: Arrest of cell cycle

c. Rieske iron-sulfur protein (RISP)

Presumed function: Arrest of mitochondrial activity

d. Histone H4 (hyperacetylated form)

Presumed function: Chromatin condensation of the macronucleus

e. Actin

Presumed function: Resorption of cilia

During *Colpoda* encystment, the expression of encystment-specific proteins is expected to occur, and most proteins expressed in vegetative cells may be silenced. Actually, mRNA levels are extremely reduced within 5h after encystment induction [11]. We analyzed the alteration of water-insoluble protein expression levels by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) and identified these proteins by LC-MS/MS analysis [11,18]. The proteins whose expression levels are modified within 12h after encystment induction are listed as follows:

a. Elongation factor 1α (EF-1α) [up-regulated 3h after encystment induction]

Presumed function: Acceleration of protein synthesis

b. ATP synthase β chain [downregulated 4h after encystment induction]

Presumed function: Disappearance of the mitochondrial membrane potential

c. Heat shock protein 60 (HSP 60) [temporarily up regulated 5h after encystment induction]

Presumed function: Molecular chaperon

d. Actin-related 49 kD protein [up-regulated 1.5h after encystment induction]

Presumed function: Chromatin remodeling

The 2-D PAGE of water-insoluble components (containing ciliary microtubules) of *C. cucullus* Nag-1 showed that the amount of tubulin is drastically reduced within several hours after the onset of encystment induction [11]. In this case, tubulin gene expression may not be downregulated but instead probably resulted from the disassembly of microtubules of ciliary axoneme. Knowledge of the signaling pathways for encystment, including those proposed by our research group, has been extensively advanced by the *Colpoda aspera* transcriptome analysis performed by Jiang et al. [19]. In the signaling pathways activated by Ca^{2+}-calmodulin, the expression of genes for AMP-activated protein kinase (AMPK), eukaryotic elongation factor-2 kinase (eEF2K), AKT (protein kinase B) and several genes for autophagy is up-regulated.

Conclusion

Although *Colpoda* is a nonpathogenic unicellular eukaryote, an understanding of molecular events in the early stages of *Colpoda* encystment is expected to help elucidate the signaling pathways leading to the encystment of pathogenic unicellular eukaryotes.

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